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Subject: Submission of synopsis of PhD work titled: "Studies on Functional Effects of β-bulge Mutations and UbEP42 Derived Double Mutations of Ubiquitin in *Saccharomyces Cerevisiae*"

Respected Sir/Madam,

Kindly accept the synopsis of my PhD work titled: "Studies on Functional Effects of β-bulge Mutations and UbEP42 Derived Double Mutations of Ubiquitin in *Saccharomyces Cerevisiae*". My date of registration is 01.09.2012 and my registration number is FoS/5/1819.

Thanking You,

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Studies on Functional Effects of β-Bulge mutations and UbEP42 Derived Double Mutations of Ubiquitin in *Saccharomyces Cerevisiae*

Synopsis

submitted

by

Prranshu Yadav

Under the guidance of

Prof. C. Ratna Prabha

Introduction

Ubiquitin is a small, 76 residue long, single domain, monomeric protein that is, as its name suggests, ubiquitously found in most types of cells across species from yeast to humans. Cells use ubiquitin as a tag by covalently attaching it to other proteins. This covalent bonding happens between the C terminal glycine of ubiquitin and a lysine on the substrate protein. This ~~is~~ process is called ubiquitination and is similar to other post translational modifications like phosphorylation, methylation and glycosylation. Ubiquitination can also involve formation of polyubiquitin chains on the substrate protein. This process is called polyubiquitination. Ubiquitin contains seven lysine residues, all of which can precipitate in ubiquitination (1, 2). Depending on which lysine residue of ~~on~~ ubiquitin covalently bonds with C terminal glycine of the next ubiquitin, polyubiquitination can be categorised into various types, which play different roles in cell. Chief among these are K48 linked polyubiquitination, which is involved in Proteasome mediated degradation of proteins that are misfolded or are no longer needed by the cell, and K63 linked polyubiquitination which is involved in various non-degradative functions like DNA repair, regulation of transcription, regulation of protein synthesis, endocytosis etc. Ubiquitination is also involved in lysosomal degradation (3, 4). Ubiquitination involves 3 types of enzymes, namely E1, E2 and E3. Ubiquitin is passed on from E1 to E2 and then from E2 to E3 through covalent linkages with these enzymes. E3 finally covalently attaches ubiquitin to a target protein that it recognises. There is an enormous diversity of E3 enzymes, with each E3 recognising and ubiquitinating a unique type or set of substrate proteins. This diversity of E3s makes ubiquitination highly specific and also gives us an opportunity to use specific E3s as drug targets to treat diseases. Ubiquitin has a highly hydrophobic core which makes it very thermostable. This high thermostability is necessary as ubiquitin is involved in stress response, which includes response to heat stress. The simple, single domain, monomeric structure of ubiquitin also makes it an ideal model to study protein folding. Moreover, the fact that the amino acid sequence of ubiquitin is highly conserved across species suggests that most of its 76 residues are under high selective pressure as they are likely involved in the structural integrity or functioning of ubiquitin (5, 6). This is also suggested from the observation that a large number of proteins contain one or more of a large variety of ubiquitin binding domains, and these domains bind various regions on the surface of ubiquitin. This invariably means that much of ubiquitin's surface residues must be conserved in order to be recognized by these

domains. It is with this background it was felt that studying structure-function relationships in ubiquitin would be useful.

In *Saccharomyces cerevisiae,* ubiquitin is expressed from 4 different genes, namely *UBI1, UBI2, UBI3* and *UBI4.* *UBI1* and *UBI2* express ubiquitin C terminally fused to L40 ribosomal protein and have an intron. *UBI3* expresses ubiquitin C terminally fused to S31 ribosomal protein and also has an intron. *UBI4* expresses a polymeric head to tail fusion of four to five ubiquitin repeats, which are post-translationally hydrolysed into ubiquitin monomers by ubiquitin C-terminal hydrolase. Unlike *UBI1, UBI2* and *UBI3* which are housekeeping genes, *UBI4* is expressed in stress conditions and helps the cell cope with stress. The stress condition is recognised by "Heat Shock Box", which is an element present upstream of the coding region of *UBI4.* (7)

To find out which residues play important roles in the structure and functions of ubiquitin, random mutations were generated in ubiquitin using error prone PCR. This process is useful for studying structure-function relationships in proteins, as it mimics the process of random mutations and natural selection in nature. Out of many mutants thus generated, one particular mutant demonstrated decreased survivability compared to wild type cells. This mutant was dubbed UbEP42 and was found to have four different mutations, namely S20F, A46S, L50P and I61T (8, 9). Now, to find out the extent to which each of these four mutations were affecting the survival of yeast, all four mutations were segregated and the isolated single mutations were studied for their structural and functional effects (9). It was found that L50P and I61T resulted in dosage dependent lethality, while S20F and A46S had no effect over survival of the organism. To test if the different combinations of these mutations have cumulative or compensatory effects, double and triple mutants were produced and structural and growth effects, survival in the presence of cycoheximide and under heat stress were studied (Mrinal Sharma's Ph. D. thesis, 2013). These studies included six double mutants, namely S20F-A46S, S20F-I61T, A46S-I61T, L50P-I61T, S20F-L50P and A46S-L50P. The lethal effects of the two mutations L50P and I61T were found to be suppressed in double mutations S20F- L50P and A46S-I61T. The present work probes through functional studies and addresses various unanswered questions to establish the 'compensatory effects' seen above with the two double mutations.

The residues Q2, E64 and S65 in ubiquitin form a G1 type β-bulge. These residues are not common in G1 β-bulges of other proteins and hence they are not the best for the stability of

β-bulge. But they are still highly conserved in the ubiquitin β-bulge across species, suggesting that they are functionally significant for ubiquitin. Comparison with other protein sequences showed that residues N, G and D are found in G1 β-bulges with much more frequency, in place of residues Q, E and S respectively, hinting that they confer more stability to the β-bulge. Therefore, to study functional and structural significance of Q2, E64 and S65, substitution mutants UbQ2N, UbE64G and UbS65D were constructed (10, 11). Structural studies using fluorescence spectroscopy and circular dichroism showed there were no significant structural changes because of the substitutions. But functional studies showed that although the substitutions had no effect on the tolerance of yeast to heat stress, they confer increased sensitivity on yeast towards cycloheximide.

There are two main objectives to this study. First, this study involve characterizing the effects of all three β-bulge mutations on:(i) Cdc28 levels, (ii) K48 linked polyubiquitination, (iii) Sensitivity towards translational inhibitors, (iv) Lysosomal degradation of uracil permease and (v) Endosomal sorting of carboxypeptidase S.

Second objective of this study is to characterize the effects of all six UbEP42 derived double mutations on: (i) Cell cycle progression, (ii) Cdc28 levels, (iii) K48 linked polyubiquitination, (iv) Ubiquitin fusion degradation pathway, (v) Sensitivity towards translational inhibitors, (vi) Lysosomal degradation of uracil permease, (vii) Endosomal sorting of carboxypeptidase S and (viii) Comparison of the performance of UbEP42 derived double mutants with triple mutants using some functional criteria.

Studies on β-Bulge Mutants of Ubiquitin

(i) Effects of β-bulge mutants on Cdc28 levels

Effect of β-bulge mutations on cell cycle was checked by observing the levels of Cdc28. Ubiquitin mutations may influence cell cycle progression as many of the cyclins involved in cell division undergo ubiquitination. Further, in a previous study from our laboratory it was observed that UbEP42 and some UbEP42 derived single mutations of ubiquitin bring down the levels of Cdc28. Cdc28 is a cyclin dependent kinase involved in the transition of cells from G1 phase to S phase during cell cycle. Levels of Cdc28 were checked in β bulge mutants and compared with that of wild type control by western blotting, using anti-Cdc28

antibody. The results indicate that there is no significant effect of β bulge mutations on Cdc28 levels. Hence, these mutations do not appear to affect the process of cell division. This is in confirmation with previous findings in our laboratory that the growth curves of β bulge mutants are identical to the wild type control.

(ii) Effects of β-bulge mutants on K48 Linked Polyubiquitination

For testing the effect of β bulge mutants on K48 linked polyubiquitination, SUB60 cells expressing wild type ubiquitin as well as those carrying ubiquitin with β-bulge mutations were grown to mid-log phase. At mid-log phase their OD was equalized and then they were lysed by sonication in normal containing protease inhibitor cocktail to prevent degradation of proteins. The protein content of the lysates was then quantified by Folin Lowry method. Lysate samples containing 50μg protein were then loaded and separated on SDS PAGE. The gels were then subjected to western blot analysis using anti-K48 antibody specific towards K48 linked polyubiquitin chains. The ponceau staining of the blot was used as loading control. The results suggest that while E64G and S65D mutations do not seem to have significant effect, Q2N mutation seems to have a negative effect on K48 linked polyubiquitination when compared to wild type.

(iii) Effects of β-bulge mutants on Sensitivity Towards Translational Inhibitors

Sensitivity of yeast towards translational inhibitors G418, hygromycin and gentamycin was tested. SUB60 is a yeast strain in which *UBI4* gene has been deleted. These cells were transformed with YEp96 plasmids carrying either the wild type ubiquitin gene or the gene for ubiquitin carrying one of the β-bulge mutations (Q2N, E64G and S65D). SUB62 strain and SUB60 transformant of plasmid with wild type ubiquitin gene were used as positive controls while untransformed SUB60 was used as negative control. These cells, along with SUB60 cells transformed with plasmids genes for β-bulge mutants of ubiquitin, were grown to mid-log phase. At mid-log phase, their OD was equalized and their serial dilutions were prepared. These serial dilutions were then deposited in the form of drops on YPD plates. These plates included a positive control containing no translational inhibitor, and three other plates containing 70μg/ml hygromycin, 40μg/ml G418 and 100 μg/ml gentamycin. The results show that β-bulge mutations increase sensitivity of cells towards G418 but not towards

hygromycin and gentamycin. Previous studies in our lab have also showed that β-bulge mutations increase sensitivity of cells towards another translational inhibitor cycloheximide. It is notable that both cycloheximide and G418 act on the elongation phase of translation while hygromycin acts by preventing transfer of t-RNA from A site to P site. Gentamycin also acts on elongation phase of translation. But inability of β-bulge mutations to increase sensitivity of cells towards it could be as studies suggest gentamycin might not act as efficiently ɵ in eukaryotes as it does in prokaryotes. This suggests that β-bulge residues might be specifically involved in the elongation phase of translation.

(iv) Effects of β-bulge mutants on Lysosomal Degradation of Uracil Permease

Uracil permease is a membrane protein of yeast, which transports uracil across the membrane. It is degraded by endocytic pathway. It is degraded only after undergoing mono or multiubiquitination. Polyubiquitins, which drive protein more effeciently towards degradation are known to be K63 linked. Doa4, ubiquitin isopeptidase, recognises K63 polyubiquitin and recycles ubiquitin. It is known that either mutation in Doa4 or UbK63R mutation decelerate uracil permease degradation (13, 14). The natural level of expression of uracil permease in yeast is so low, that it cannot be easily detected in western blot. Hence, the cells had to be transformed with p352 plasmid, that also carries Fur4 gene for uracil permease, and hence additional uracil permease is expressed in the cell. After this, the levels of uracil permease were checked in mutants and wild type control by western blotting, using anti-Fur4 antibody, that recognizes uracil permease. From the results of western blotting, it was apparent that there is no measurable effect of β-bulge mutations on lysosomal degradation of uracil permease. Western blot of uracil permease shows that β-bulge mutations do not significantly affect the lysosomal degradation of uracil permease.

(v) Effects of β-bulge mutants on Endosomal Sorting of Carboxypeptidase S

Carboxypeptidase S or CPS, is a proteolytic enzyme in yeast. It is known to be a cargo of multivesicular endosomes, and its sorting into multivesicular endosomes depends on K63 polyubiquitination (12). To test the effects of β-bulge mutations on endosomal sorting of carboxypeptidase S, henceforth referred to as CPS, cells were first transformed with a plasmid expressing CPS genetically fused to the gene for GFP (green fluorescent protein).

The cells were then treated with FM4-64, which is a dye that specifically stains the membranes of vacuoles and multivesicular endosomes. The cells thus treated and fixed were then observed by confocal fluorescence microscopy. The results show from the fluorescence intensity that the amount of overlap between the green fluorescence of GFP-CPS and the red fluorescence of FM 4-64 is indistinguishable between wild type cells and those containing β-bulge mutations. This suggests that β-bulge mutations do not have any significant effect on endosomal sorting of carboxypeptidase S.

Studies on UbEP42 Derived Double Mutants

(i) Effects of UbEP42 Derived Double Mutants on Cell Cycle

To study the effect that UbEP42 derived double mutations have on cell cycle, cell cycle in these mutants is going to be analysed by fluorescence activated cell sorting (FACS).

(ii) Effects of UbEP42 Derived Double Mutants on Cdc28 Levels

To check the effect of UbEP42 derived double mutations on cell cycle, levels of Cdc28 were checked in cells using western blot. For this, cells were grown to log phase and their OD was equalized, following which they were lysed by sonication in normal saline containing protease inhibitor cocktail. The protein content of lysates was determined by Folin Lowry method and samples containing 40 μg/ml of protein were loaded and run on SDS-PAGE. The gel was then subjected to western blotting using an antibody specific for Cdc28. The results indicate that mutants S20F-A46S, S20F-L50P and A46S-I61Tshow Cdc28 levels similar to wild type cells, while S20F-I61T, A46S-L50P and L50P-I61T show decreased levels of Cdc28 when compared to wild type cells. The three mutants S20F-A46S, S20F-L50P and A46S-I61T show compensatory effects.

Effects of UbEP42 Derived Double Mutants on K48 Linked Polyubiquitination

For testing the effect of UbEP42 derived double mutants on K48 linked polyubiquitination, the same protocol as the one used in case of β-bulge mutants was used. SUB60 cells expressing wild type ubiquitin as well as those carrying ubiquitin with UbEP42 derived double mutations were grown to mid-log phase. At mid-log phase their OD was equalized and then they were lysed by sonication in normal saline in the presence of protease inhibitor

cocktail to prevent degradation of proteins. The protein content of the lysates was then quantified by Folin Lowry method. Lysate samples containing 50μg protein were then loaded and resolved on SDS PAGE. The gel was then subjected to western blot analysis using anti-K48 antibody, that is specific towards K48 linked polyubiquitin chains. The ponceau S staining was done on this blot as loading control. The results suggest that mutants S20F-I61T, A46S-L50P and L50P-I61T have a negative effect on K48 linked polyubiquitination when compared to wild type, while the three mutants S20F-A46S, S20F-L50P and A46S-I61T seem to have a positive effect.

Effects of UbEP42 Derived Double Mutants on Sensitivity Towards Translational Inhibitors

The protocol for testing effect of UbEP42 derived double mutations on sensitivity towards translational inhibitors was the same as that used in case of β-bulge mutants. Cells were transformed with YEp96 plasmids carrying either the wild type ubiquitin gene or the gene for ubiquitin carrying one of the UbEP42 double mutations. These cells were grown to log phase. At log phase, their OD was equalized and their serial dilutions were prepared. These serial dilutions were then deposited in the form of drops on YPD plates. The results show that in presence of 2μg/ml canavanine, S20F-L50P and A46S-I61T show survival similar to wild type cells. In presence of 50 μg/ml G418, mutants S20F-L50P, S20F-I61T, A46S-L50P and A46S-I61T show increased compared to wild type cells. In presence of 80 μg/ml gentamycin, the mutants appear to survive like wild type cells. In presence of 40 μg/ml hygromycin, S20F-A46S, A46S-I61T and L50P-I61T show decreased survival compared to wild type cells. And in presence of 0.75 μg/ml of tunicamycin, mutants appear to survive ~~be~~ just as well as wild type cells.

Effects of UbEP42 Derived Double Mutants on Ubiquitin Fusion Degradation Pathway

To test effects of UbEP42 derived double mutations on ubiquitin fusion degradation pathway, cells were co-transformed with YEp96 plasmid expressing either double mutant or wild type ubiquitin and pUb23 plasmid carrying ubiquitin fusion protein of ubiquitin and β-galactosidase with N-terminal amino acid residue X as either Met (M) or Pro (P) in Ub-X-βgal. There are two sets of pUb23 plasmids. In one set, the X position is occupied by methionine or Met, hence Ub-M-βgal, and in other set the X position is occupied by proline or Pro, and hence Ub-P-βgal. If methionine is present at the N-terminal of β-galactosidase, it is not degraded by UFD pathway, while proline promotes degradation of β-galactosidase by UFD pathway when present in position X. After co-transformation, cells were grown to mid-

log phase and their OD equalized. The cells were then lysed and protein content of lysates was determined by Folin Lowry method. Samples containing 40μg of protein were then loaded and resolved on SDS-PAGE. The gel was then subjected to western blot analysis using anti-β-galactosidase antibody. The controls used were β-galactosidase, M- βgal and P-βgal. The results show that in three UbEP42 double mutants, namely S20F-I61T, A46S-L50P and L50P-I61T, the amount of β-galactosidase degraded from Ub-P-βgal is comparable to that from Ub-M-βgal. This suggests that S20F-I61T, A46S-L50P and L50P-I61T mutations affect the ability of proline residue to channelize degradation by UFD pathway. Notably, these are the same three mutations that affect K-48 linked polyubiquitination and lysosomal degradation of uracil permease. However, the mutations S20F-L50P, A46S-L50P and A46S-I61T behaved like wild type cells with respect to degradation by UFD pathway.

Effects of UbEP42 Derived Double Mutants on Lysosomal Degradation of Uracil Permease

To test the effects of UbEP42 derived double mutations on lysosomal degradation of uracil permease, cells were first transformed with p352 plasmid to produce extra uracil permease and make it easy to observe with western blot. Cells were grown to mid-log phase and after being treated with 100μg/ml cycloheximide for 20 minutes. Cycloheximide was used to halt further synthesis of uracil permease. The cells were lysed with sonication in normal saline in the presence of protease inhibitor cocktail. Two sets of cells were lysed. One set was lysed right after treatment with cycloheximide and the other set was lysed 90 minutes after treatment with cycloheximide. The protein content of the lysates was then estimated by Folin Lowry method. Lysate samples containing 40μg protein were then loaded and resolved on SDS PAGE. The gel was then subjected to western blot analysis using an anti Fur4 antibody,that is the antibody specific for uracil permease. The results show that in mutants S20F-I61T, A46S-L50P and L50P-I61T, the amount of uracil permease degraded 90 minutes after treatment with cycloheximide was significantly less than the wild type cells. However, the mutations S20F-L50P, A46S-L50P and A46S-I61T behaved like wild type cells with respect to uracil permease levels.

Effects of UbEP42 Derived Double Mutants on Endosomal Sorting of Carboxypeptidase S

To test effects of UbEP42 derived double mutations on endosomal sorting of carboxypeptidase S (CPS), the same protocol used in case of β-bulge mutants was used. Briefly, SUB60 cells were first transformed with a plasmid expressing CPS genetically fused

to GFP. The cells were then treated with FM 4-64, which is a dye that specifically stains the membranes of vacuoles and multivesicular endosomes. The cells thus treated and fixed were then observed by confocal fluorescence microscopy. The results show the difference in the amount of overlap between the green fluorescence of GFP-CPS and the red fluorescence of FM4-64 is indistinguishable between wild type cells and those containing UbEP42 derived double mutations, as is the case with β-bulge mutations. This suggests that like β-bulge mutations, UbEP42 double mutations also do not have any significant effect on endosomal sorting of carboxypeptidase S.

Comparison of the performance of UbEP42 derived double mutants with triple mutants will be carried out to see if triple mutants also produce compensatory effects, using some functional criteria such as cell cycle progression, resistance to antibiotics and sorting of carboxypeptidase S.

Conclusion

In conclusion, two different classes of ubiquitin mutants generated in our lab have been studied in this work. These two classes are the β-bulge mutants Q2N, E64G and S65D, and UbEP42 derived mutants consisting of various combinations of four different mutations, namely S20F, A46S, L50P and I61T. Among the UbEP42 derived mutants, this involves study of the six double mutants, namely S20F-A46S, S20F-I61T, A46S-I61T, L50P-I61T, S20F-L50P and A46S-L50P, and the four triple mutants derived from UbEP42 are also going to be studied. Among the β-bulge mutants, an interesting finding is that β-bulge residues might be involved specifically in the elongation step of translation. This is because β-bulge mutants exhibit heightened sensitivity towards G418 and cycloheximide, both of which act on the elongation phase of translation, but not against hygromycin, which acts by preventing transfer of t-RNA from A site to P site. Previous studies have also shown that some ribosomal proteins like L28 undergo K63 linked polyubiquitination. The β-bulge residues lie adjacent to K63 residue. This might explain the effect of β-bulge mutations on translation. In the study of UbEP42 derived double mutations, an interesting trend has been observed regarding the mutants S20F-L50P and A46S- I61T. These two double mutants display compensatory effect on K-48 linked polyubiquitination, UFD pathway, and lysosomal degradation of uracil permease, and show higher levels of Cdc28 similar to wild type cells. It is likely that their compensatory effect seen in these combinations of mutations is a result of restoration of ubiquitin structure leading to normal levels of K48 linked polyubiquitination, lysosomal degradation and Cdc28 levels. To further study how these altered Cdc28 levels

affect cell cycle, FACS analysis of UbEP42 double mutants will be done. Along with this, studies are also going to be done on UbEP42 derived triple mutants, to see if the effects observed in double mutants in this work and in single mutants in previous studies can be observed in triple mutants as well.

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